AD_____
(Leave blank)

Award Number: W81XWH-07-1-0144

TITLE: Detection of Prostate Cancer Progression by Serum DNA Integrity

PRINCIPAL INVESTIGATOR: Dave S.B. Hoon, MSc, Ph.D.

CONTRACTING ORGANIZATION: John Wayne Cancer Institute
                          at St. John's Health Center
                         Santa Monica, CA 90404

REPORT DATE: April 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
              Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

   ☒ Approved for public release; distribution unlimited

   ☐ Distribution limited to U.S. Government agencies only;
      report contains proprietary information

The views, opinions and/or findings contained in this report are those of
the author(s) and should not be construed as an official Department of the
Army position, policy or decision unless so designated by other
documentation.

REPORT DOCUMENTATION PAGE

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
30-04-2009	ANNUAL	1 Apr 2008 – 31 Mar 2009

4. TITLE AND SUBTITLE

Detection of Prostate Cancer Progression by Serum DNA Integrity

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0144

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dave S.B. Hoon, Ph.D.; Email: hoon@jwci.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

John Wayne Cancer Institute
2200 Santa Monica Boulevard
Santa Monica, CA 90404

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; Distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Toward the objective/hypothesis of obtaining additional diagnostic and prognostic insight through the analysis of tumor-related DNA integrity and methylation in patient serum, we continue to procure prostate patient specimens and develop optimized assessment assays. We have utilized prostate cell lines and tumor specimens for the assay optimization and validation. In a study of 57 prostate patients, we found significant differences of LINE1 status in patients compared to normal healthy male. The data is being assembled in a manuscript for submission to a peer reviewed journal. In the coming year, more PCA patients will be accrued as well as further accrual of serum at defined treatment intervals. Assays for the markers will be carried out for LINE1 and other promising biomarkers for clinical utility validation. We will also continue our efforts in discovering additional clinically relevant DNA biomarkers.

15. SUBJECT TERMS
Circulating DNA, serum, prostate cancer, methylation, PCR

16. SECURITY CLASSIFICATION OF: Unclassified			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	22	19b. TELEPHONE NUMBER (include area code)

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION

Toward the objective/hypothesis of obtaining additional diagnostic and prognostic insight through the analysis of tumor-related DNA integrity and methylation in patient serum, we continue to procure prostate patient specimens and develop optimized assessment assays. We have utilized prostate cell lines and tumor specimens for the assay optimization and validation.

In this second program year, we have focused on the following: 1) Optimized *Alu* DNA integrity assay in cell lines and tumors. Challenges with the multifocality of the tumor were overcome to establish a valid assessment assay using a quantitative direct assay to determine its clinical utility; we were not able to assess the serum with this assay during the year. 2) LINE 1 and uLINE 1 assays were optimized and used in the assessment of serum from 57 PCa patients and 25 normal healthy male with significant findings. 3) We will determine the combined clinical utility of the three circulating serum tumor-related DNA markers in monitoring patients' response to treatment. The long-term goal is to validate the clinical utility of these markers.

In the second year of the proposal period, we continue to accrue PCa patients and normal donor serum for the study with an IRB approved protocol. As outlined in Task 1, samples are continuing to be collected, coded, logged into a database, processed for serum and quality assured. Task 1 will be ongoing until we reach our proposed sample numbers. Some of these samples have been used in pilot studies of the various assays proposed.

Optimization of methylation of biomarkers has been performed on normal serum, tissues and cell line dilution studies. Available paraffin-embedded (PE) PCa tissue samples were H&E stained, laser microdissected with assistance from a pathologist and assessed using our methylation assay to verify results. We have demonstrated proficiency in DNA extraction from micro-dissected tumor tissues and adjacent normal tissues. We have employed our new technique of on-slide bisulfite technique to assess very small tumor lesions. This allows analysis without loss of DNA during bisulfite treatment of paraffin embedded tissue sections. This has allowed us assessment of a small number of tissue sections which was previously very difficult. Multifocal and single tumor lesions were assessed for presence of methylation biomarkers. Tumor heterogeneity of lesions within an individual patient was assessed in detail to determine level of significance among biomarkers. New biomarkers have been assessed and optimized such as estrogen receptor, WNT pathways, and RASSF1A. We have demonstrated heterogeneity in multifocal lesions in patients with radical prostectomies. These results are being used to assess the prominent biomarkers in serum.

As reported in our last progress report, we have developed the uLINE1 AQAMA assay for the assessment of PCa patient serum. The results of the pilot study of 18 prostate cancer PE tissues with matched adjacent normal tissues led to the optimization of the assay for serum assessment. As reported, we had great success in establishing the sensitivity and specificity. Although the sample size is still small at 57 prostate cancer patients, the LINE 1 assay has demonstrated its usefulness in discriminating between serum samples of cancer patients and normal male healthy volunteers.

We have devised several ways of interrogating the clinical samples using LINE1 assay. We first investigated the LINE1 hypomethylation index to differentiate the clinical specimens of patients with various stages of disease. **Figure 1** shows the mean hypomethylation index value of patient serum samples were significantly higher than normal healthy males($p=0.002$). Then we quantified the LINE1 copies found in serum samples and found that the difference between normal males and Stage IV patients was significant ($p=0.019$)(**Figure 2**). Lastly, we investigated the copy number of unmethylated LINE1 in the serum and again found the difference between Stage IV patients and normal healthy male to be significant ($p<0.0001$)(**Figure 3**). Table 1 contains the clinicopathological characteristics of the PCa patients studied

Figure 1

| | Normal
N=25
Ave.=0.066 | Stage I, II, III
N=11
Ave.=0.095 | Stage IV
N=46
Ave.=0.090 |

P=0.002

p=0.0002

Figure 2

| | Normal
N=24
Ave.=1.57x10*4 | Stage I, II, III
N=11
Ave.=2.09x10*4 | Stage IV
N=41
Ave.=4.26x10*4 |

p=0.019

Figure 3

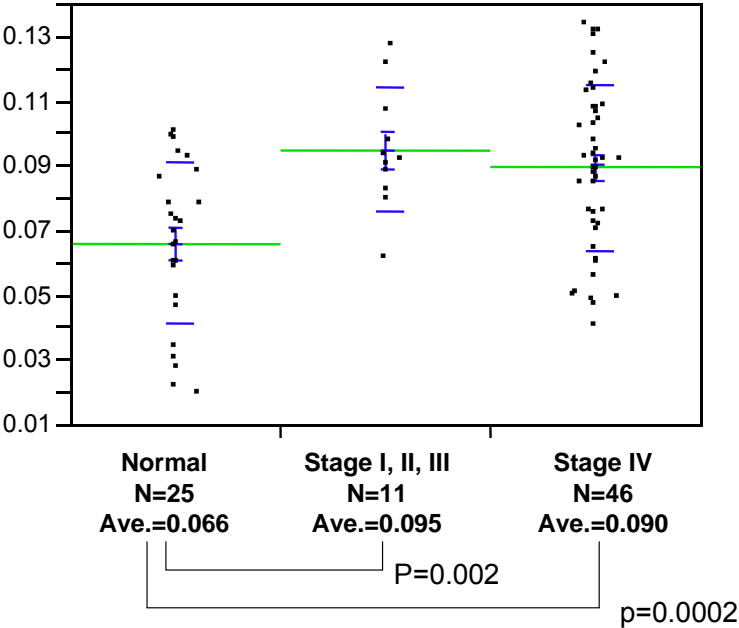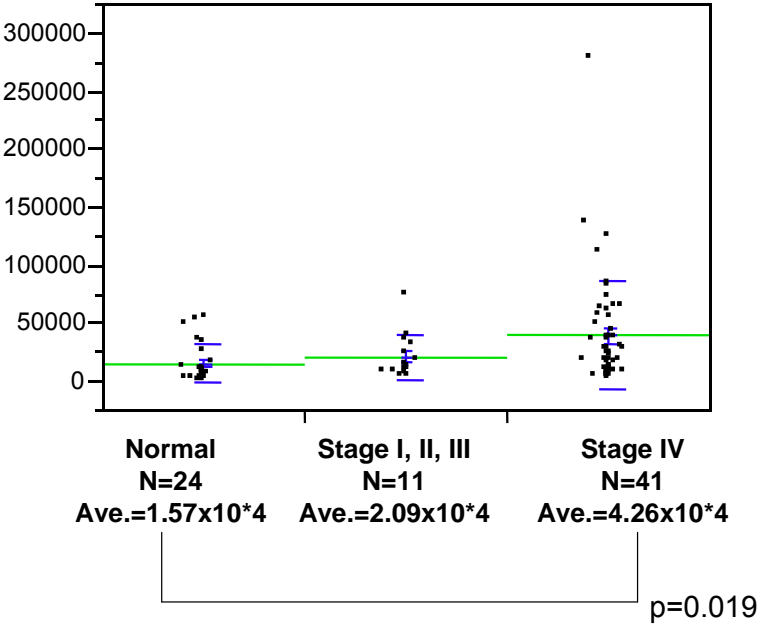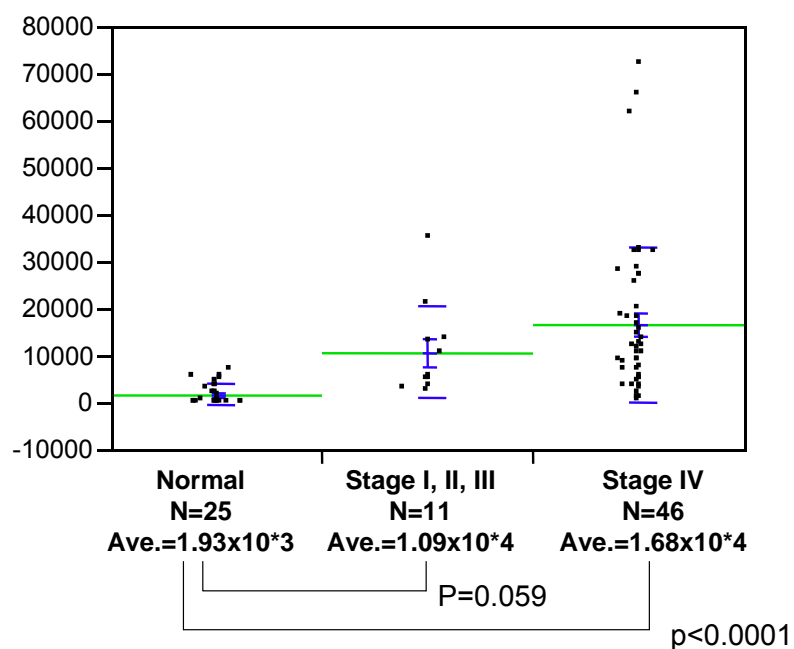| | Normal
N=25
Ave.=1.93x10*3 | Stage I, II, III
N=11
Ave.=1.09x10*4 | Stage IV
N=46
Ave.=1.68x10*4 |

P=0.059

p<0.0001

Table 1

Table 1. Clinicopathological characteristics of primary prostate cancers (n = 57)

Variable		Number of patients (%)	
Sex			
	male	57	(100)
	female	0	(0)
AJCC	stage		
	I	2	(4)
	II	4	(7)
	III	5	(9)
	IV	46	(81)
Gleason	Score		
	5	4	(9)
	6	1	(2)
	7	22	(48)
	8	7	(15)
	9	11	(24)
	10	1	(2)
PSA			
	<= 4 ng/ml	13	(23)
	>4 ng/ml	43	(77)
	mean	110.6	
Age	mean	68.1	

We plan to continue our efforts both in the sample accrual, new biomarker development and assay sensitivity improvement. We are currently assembling the data collected so far for the LINE1 assay in a manuscript for submission to a peer review journal within several months. Refer to the grant tasks itemization and list for our future direction.

KEY RESEARCH ACCOMPLISHMENTS

1. PCa patient accrual for serum.
2. Normal healthy donor serum accrual for serum.
3. Database constructed and utilized to keep track of samples.
4. Clinical data recorded and collected in the database.
5. Blood is processed for serum; DNA is extracted and quantified
6. LINE1 methylation biomarkers in serum are detected.
7. LINE1 methylation biomarkers assessed and optimized for detection in serum.
8. LINE1 methylation biomarkers optimized for specificity and sensitivity.
9. LINE1 data showed significance in clinical samples.
10. Preparation of manuscript on LINE1 assay in patient serum

REPORTABLE OUTCOMES

We have one manuscript published during this grant period.

Sunami E, Shinozaki M, Higano CS, Wollman R, Dorff TB, Tucker SJ, Martinez SR, Mizuno R, Singer FR, **Hoon DS.** Multimarker circulating DNA assay for assessing blood of prostate cancer patients.Clin Chem. 2009 Mar;55(3):559-67. Epub 2009 Jan 8.Erratum in: Clin Chem. 2009 Jun;55(6):1258. Mizuno, Ryuich [added].

CONCLUSIONS

The planned studies are being conducted according to the approved schedule as delineated in the protocol. The studies are very important in that having developed a sensitive molecular blood test for prostate cancer patients will improve current approaches of watchful waiting for disease recurrence. We have made major strides in developing biomarker detection in paraffin-embedded tissue sections of single and multilesion prostate cancers. This has aided us significantly in identifying candidate serum methylation biomarkers.

REFERENCES

None.

APPENDICES

Sunami E, Shinozaki M, Higano CS, Wollman R, Dorff TB, Tucker SJ, Martinez SR, Mizuno R, Singer FR, **Hoon DS**. Multimarker circulating DNA assay for assessing blood of prostate cancer patients.Clin Chem. 2009 Mar;55(3):559-67. Epub 2009 Jan 8.Erratum in: Clin Chem. 2009 Jun;55(6):1258. Mizuno, Ryuich [added].

The above manuscript is attached below.

Multimarker Circulating DNA Assay for Assessing Blood of Prostate Cancer Patients

Eiji Sunami,[1] Masaru Shinozaki,[1] Celestia S. Higano,[3] Robert Wollman,[4] Tanya B. Dorff,[5] Steven J. Tucker,[6] Steve R. Martinez,[1] Frederick R. Singer,[2] and Dave S.B. Hoon[1*]

BACKGROUND: Prostate cancer (PCa) detection using serum-based prostate specific antigen (PSA) is limited by frequent false-positive and -negative results. Genetic aberrations such as allelic imbalance (AI) and epigenetic changes such as promoter hypermethylation have been detected in circulating DNA of cancer patients. We hypothesized that circulating multimarker DNA assays detecting both genetic and epigenetic markers in serum would be useful in assessing PCa patients.

METHODS: We assayed blood from healthy male donors (n = 40) and 83 patients with American Joint Cancer Committee (AJCC) stage I–IV PCa. DNA was assayed for AI of 6 genome microsatellites. We assessed methylation of *RASSF1*, *RARB2*, and *GSTP1* using a methylation-specific PCR assay and analyzed the sensitivity of each assay for the detection of genetic or epigenetic changes in circulating DNA. The relation between circulating tumor-related DNA detection and prognostic factors was investigated.

RESULTS: The proportion of patients demonstrating AI for ≥1 marker was 47% (38 of 81 patients). Methylation biomarkers were detected in 24 of 83 patients (28%). By combining 2 DNA assays, the number of PCa patients positive for ≥1 methylated or LOH marker increased (52 of 83; 63%). The combined assays detected PCa in 15 of 24 patients (63%) with normal PSA concentrations. The combination of the DNA assays detected the presence of PCa regardless of AJCC stage or PSA concentration. Combination of the DNA and PSA assays gave 89% sensitivity.

CONCLUSIONS: This pilot study demonstrates that the combined circulating DNA multimarker assay identifies patients with PCa and may yield information independent of AJCC stage or PSA concentration.

© 2008 American Association for Clinical Chemistry

Prostate cancer (PCa)[7] is the most frequently diagnosed malignancy in men in the US. The 2007 estimates from the American Cancer Society predicted 218 890 new cases of PCa and 27 050 deaths *(1)*. Although serum concentration of prostate-specific antigen (PSA) remains important for prognosis and diagnosis *(2)*, the PSA assay is not particularly accurate *(3–6)*: up to 80% of patients with an increased PSA concentration will have negative biopsy results, and up to 25% of patients with a normal serum PSA (<4 μg/L) will ultimately have biopsy evidence of PCa *(7)*. Highly specific, minimally invasive techniques that are cost effective for screening and monitoring would facilitate earlier diagnosis of PCa and may also reduce the number of unnecessary biopsies.

The progression of PCa and other malignancies is characterized by increased genetic and epigenetic aberrations not usually found in normal DNA *(8–11, 12)*. Of particular interest is allelic imbalance (AI) in a genomic region, which may affect cellular regulatory genes, tumor suppressor genes, or oncogenes *(13, 14)*. An equally important epigenetic change is hypermethylation of tumor-related genes in the promoter region, which can turn off genes and significantly affect the initiation and progression of neoplasia *(15, 16)*. Both genetic and epigenetic changes can be measured in tissue or body fluids, but only the latter specimen type is useful for serial assessments and minimally invasive screening *(17–19)*. Thus far, however, most investigations of DNA biomarkers in patients with PCa have focused on tissue assessment and have measured a sin-

[1] Department of Molecular Oncology and [2] the Breast and Endocrine Program, John Wayne Cancer Institute, Saint John's Health Center, Santa Monica, CA; [3] Department of Medicine, Division of Oncology, University of Washington and Seattle Cancer Care Alliance, Seattle, WA; [4] Department of Radiation Oncology, Saint John's Health Center, Santa Monica, CA; [5] The Angeles Clinic and Research Institute, Santa Monica, CA; [6] Pacific Cancer Center, Paragon, Singapore.
* Address correspondence to this author at: Department of Molecular Oncology, John Wayne Cancer Institute, 2200 Santa Monica Blvd., Santa Monica, CA

[7] Nonstandard abbreviations: PCa, prostate cancer; PSA, prostate-specific antigen; AI, allelic imbalance; AJCC, American Joint Cancer Committee; CAE, capillary array electrophoresis; MSP, methylation-specific PCR; LOH, loss of heterozygosity.

gle type of genetic aberration using 1 platform *(13, 20–35)*.

In this pilot study, we hypothesized that a dual-assay system based on both genetic and epigenetic alterations in multiple microsatellite and methylation markers would be more sensitive for detection of PCa than a single-assay, single-marker approach. We assessed AI and hypermethylation in circulating DNA from the serum of patients with American Joint Cancer Committee (AJCC) stage I, II, III, and IV PCa. AI at loci on chromosomes 6q, 8p, 9p, 10p, and 18q *(13, 20–26)* was evaluated by assessing loss of DNA microsatellites. The epigenetic biomarkers evaluated were 3 tumor suppressor genes that are frequently hypermethylated in PCa: *GSTP1* (glutathione S-transferase π1),[8] *RARB2* (retinoic acid receptor β, variant 2), and *RASSF1* [Ras association (RalGDS/AF-6) domain family member 1] *(28–34)*.

Materials and Methods

SPECIMEN COLLECTION AND DNA ISOLATION

Institutional Review Board approval was obtained from John Wayne Cancer Institute at Saint John's Health Center, Santa Monica, CA, and the University of Washington, Seattle, WA. All patients provided signed, written informed consent to participate in this study. We enrolled 83 patients receiving treatment for PCa at John Wayne Cancer Institute, The Angeles Clinic and Research Institute, and the University of Washington. Patient characteristics are presented in Table 1. PCa patients represented AJCC stage I (n = 3), stage II (n = 15), stage III (n = 7), or stage IV (n = 58).

We collected 10 mL blood from each patient in a serum separator tube and processed it immediately as follows: separated by centrifugation (1000*g*, 15 min), filtered through a 13-mm serum filter (Fisher Scientific), separated into aliquots, and cryopreserved at −80 °C. DNA was extracted and processed from serum as previously described *(36)*. Briefly, 500 μL of serum was diluted with 0.9 mol/L NaCl$_2$, SDS, and proteinase K (Qiagen) and incubated at 50 °C for 3 h. An equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) was then added and the sample was vortex-mixed vigorously. After centrifugation at 1000*g* for 10 min, the aqueous layer was collected. DNA was precipitated using pellet paint NF coprecipitant (Novagen) and isopropanol *(36)*. Extracted DNA from serum was

Table 1. Patient characteristics.

	n
All patients	83
AJCC stage	
I/II	18
III	7
IV	58
Gleason score	
5	3
6	8
7	28
8	14
9	16
10	1
PSA, ng/mL	
≤4	24
>4	57
Mean	22.4
Mean age, years	70.4

subjected to sodium bisulfite modification *(17)*. Briefly, DNA from 500 μL of serum was supplemented with 1 μg salmon sperm DNA (Sigma) and denatured in 0.3 mol/L NaOH for 3 min at 95 °C. Overall, 550 μL of a 2.5 mol/L sodium bisulfite and 125 mmol/L hydroquinone solution were added. Samples were incubated in the dark for 3 h at 60 °C. Salts were removed using the Wizard DNA Clean-Up System (Promega) and desulfonated in 0.3 mol/L NaOH at 37 °C for 15 min. Modified serum DNA was prepared and stored at −30 °C. We performed DNA quantification on all serum specimens using the PicoGreen quantification assay (Molecular Probes) *(17)*.

Genomic DNA isolated from the peripheral blood lymphocytes of all PCa patients served as internal controls for AI. Additionally, DNA isolated from the serum of 40 healthy donors served as normal controls for the assessment of tumor-related gene hypermethylation. DNA quantification was performed using the PicoGreen assay (Molecular Probes) according to manufacturer directions. Samples were run in triplicate, and the results are expressed as the mean concentration for each patient.

MICROSATELLITE ANALYSIS

The following microsatellite markers and their corresponding chromosomal loci were evaluated for AI: D6S286 at 6q14, D8S261 at 8p22, D8S262 at 8p23, D9S171 at 9p21, D10S591 at 10p15, and D18S70 at 18q23. Forward primer sets were labeled with WellRed

---

[8] Human genes: *GSTP1*, glutathione S-transferase π1; *RARB2*, retinoic acid receptor β, variant 2; *RASSF1*, Ras association (RalGDS/AF-6) domain family member 1; *CDKN2A*, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) (alias, *p16*); *ARF*, ADP-ribosylation factor; *MGMT*, O-6-methyl-guanine-DNA methyltransferase.

phosphoramidite-linked dye (Research Genetics, Inc.). PCR reactions were performed in 10-μL volumes with 1 μL template for 40 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by a final extension of 7 min at 72 °C, as described (18). We performed post-PCR product separation using capillary array electrophoresis (CAE) (CEQ 8000XL; Beckman Coulter Inc.). AI was defined when a ≥40% reduction in serum DNA allele peak intensity was noted compared to the respective patient's normal lymphocyte DNA allele peak using CEQ 8000XL software.

BISULFITE MODIFICATION AND METHYLATION-SPECIFIC PCR

Extracted DNA from serum was subjected to sodium bisulfite modification (17). DNA from 500 μL serum was supplemented with 1 μg salmon sperm DNA (Sigma) and denatured in 0.3 mol/L NaOH for 3 min at 95 °C. Overall, 550 μL of a 2.5 mol/L sodium bisulfite and 125 mmol/L hydroquinone solution were added. Samples were incubated in the dark for 3 h at 60 °C. Salts were removed using the Wizard DNA Clean-Up System (Promega) and desulfonated in 0.3 mol/L NaOH at 37 °C for 15 min. Modified serum DNA was prepared and stored at −30 °C.

We assessed the methylation status of *GSTP1*, *RARB2*, and *RASSF1* using methylation-specific PCR (MSP) using 2 sets of fluorescent-labeled primers specifically designed to amplify methylated and unmethylated DNA sequences, respectively. In total, 100 ng bisulfite-modified DNA was subjected to PCR amplification in a final reaction volume of 10 μL containing PCR buffer, 2.5–4.5 mmol/L MgCl$_2$, dNTPs, 0.3 μmol/L primers, 0.5 U Ampli*Taq* gold polymerase (Applied Biosystems), and 50 pmol of each forward (F) and reverse (R) primer set for methylated (M) and unmethylated (U) primers as follows: *RARB2*, (M) F-GAACGCGAGCGATTCGAGT and R-GACCAATCCAACCGAAACG, (U) F-GGATT GGGATGTTGAGAATGT and R-CAACCAATCCAAC CAAAACAA; *RASSF1*, (M) F-GTGTTAACGCGTTG CGTATC and R-AACCCCGCGAACTAAAAACGA, (U) F-TTTGGTTGGAGTGTGTTAATGTG and R-CAAA CCCCACAAACTAAAAACAA; *GSTP1*, (M) F-TTCGG GGTGTAGCGGTCGTC and R-GCCCCAATACTAA ATCACGACG, (U) F-GATGTTTGGGGTGTAGTGG TTGTT and R-CCACCCCAATACTAAATCACAACA. We carried out PCR after optimizing annealing temperatures for each primer set (60 °C for *RASSF1* and 59 °C for *RARB2* and *GSTP1*). An initial 10-min incubation at 95 °C was followed by 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30 s, and a final hold for 7 min at 72 °C. Sodium bisulfite–modified DNA from healthy donors' lymphocytes and ϕ-29–treated genomic DNA served as unmethylated negative control DNA for each PCR reaction, whereas lymphocytes treated with *Sss*I Methylase (New England Biolabs) followed by sodium bisulfite modification served as positive methylated control DNA (37). Molecular biology–grade water without DNA served as a reagent control. Post-PCR products were separated and assessed using CAE. CAE was performed in a 96-well microplate format, combining 1 μL of each methylated and unmethylated PCR reaction product with 40 μL loading buffer and 0.5 μL dye-labeled size standard (Beckman Coulter) in a single well. Forward MSP primers were labeled with D4pa dye, whereas forward unmethylated specific primers were labeled with D2pa dye. This permitted discrimination of the 2 peaks for direct comparison of post-PCR methylated and unmethylated products in a single run. CEQ 8000XL software was used to determine PCR product signal intensity.
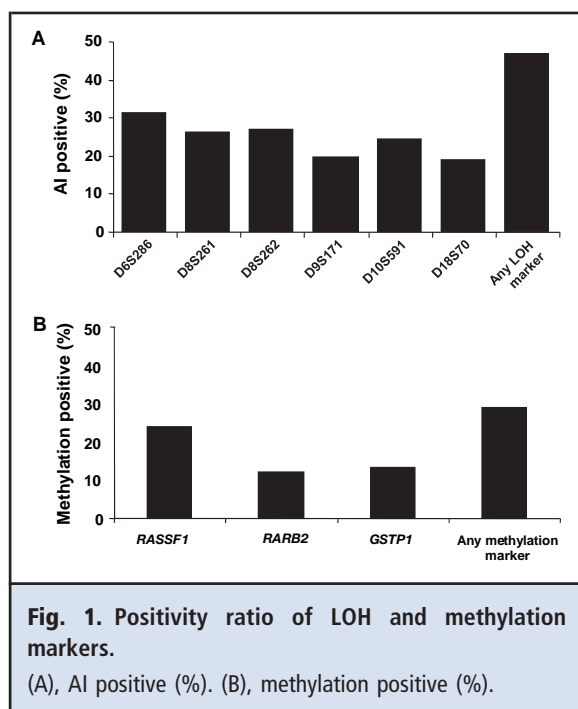
STATISTICAL ANALYSIS

Descriptive statistics, such as mean, SD, median, frequency, and percentage, were used to summarize patient characteristics, AI status, and gene hypermethylation status. We used ANOVA with the Dunnett method for posthoc testing (for continuous variables) and the χ^2 test (for categorical variables) for comparing clinical factors among tumors demonstrating AI vs heterozygous alleles or hypermethylation vs nonmethylation. Statistical analysis was by JMP software (SAS). All statistical tests were 2-sided, with significant *P* values <0.05.

Results

LOSS OF HETEROZYGOSITY IN CIRCULATING DNA

We evaluated the presence of AI in serum circulating tumor-associated DNA from PCa patients using 6 microsatellite markers known to demonstrate frequent loss of heterozygosity (LOH) in PCa. The number of informative patient samples for any 1 microsatellite marker ranged from 33% (27 informative patient samples) for D8S261 to 70% (58 informative patient samples) for D18S70. The most common microsatellite for AI was D6S286, which was detected in 14 of 45 (31%) informative patient samples. This was followed by D8S262 (11 of 41, 27%), D8S261 (7 of 27, 26%), D10S591 (11 of 45, 24%), D9S171 (9 of 46, 20%), and D18S70 (11 of 58, 19%). Of patient samples with at least 1 informative marker (81 cases), 20 (25%) had only 1 LOH marker detected in the serum sample, 13 samples (16%) had 2 LOH markers detected, and 5 samples (6%) had 3 or more LOH markers detected. The proportion of informative patient samples demonstrating AI for ≥1 marker was 47% (38 of 81 patient samples; Fig. 1A). Healthy male donors (n = 40) did not have these specific circulating LOH markers in their sera. Detection of AI correlated with AJCC PCa

Fig. 1. Positivity ratio of LOH and methylation markers.

(A), AI positive (%). (B), methylation positive (%).

stage overall. There were no significant differences in AI status between individual stages.

HYPERMETHYLATION OF TUMOR-RELATED GENES

We assessed serum circulating DNA from PCa patients for hypermethylation of the tumor-related genes *RASSF1*, *GSTP1*, and *RARB2* (Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue3), which have shown to be hypermethylated in primary prostate tumors *(28–34)*. The most frequently detected methylated gene circulating in serum was *RASSF1*, detected in 20 of 83 (24%) patient samples. *RARB2* and *GSTP1* were detected in 10 of 83 (12%) and 11 of 83 (13%) patient samples, respectively. The proportion of patient samples demonstrating hypermethylated circulating DNA of only 1 tumor-related biomarker was 17% (14 of 83), whereas 4% (3 of 83) and 8% (7 of 83) demonstrated 2 and 3 hypermethylated DNA markers, respectively. The combination of all 3 methylation markers increased the detection rate to 24 of 83 (29%) PCa patients (Fig. 1B). Healthy male donors did not have these specific circulating methylated DNA markers in their sera under optimal assay conditions.

CIRCULATING LOH AND METHYLATED DNA CORRELATION WITH SERUM PSA

To assess the clinical relevance of AI and methylation, we correlated LOH and hypermethylation with serum PSA. We assessed blood drawn for serum LOH and for serum PSA in 81 of 83 (98%) patients. No significant differences were seen in the mean serum PSA concentrations among patients with AI of D9S171, D10S591, D8S261, D6S286, D8S262, and D18S70 and heterozygous patients. The mean serum PSA concentration in patients with hypermethylated tumor-related genes (*RASSF1*, *RARB2*, and *GSTP1*) was significantly higher than in patients without hypermethylated genes (*P* = 0.002, 0.017, and <0.0001, respectively). Similarly, patients with ≥1 hypermethylated tumor-related gene had serum PSA concentrations that were significantly higher than patients without circulating hypermethylated DNA (*P* = 0.002; Fig. 2).

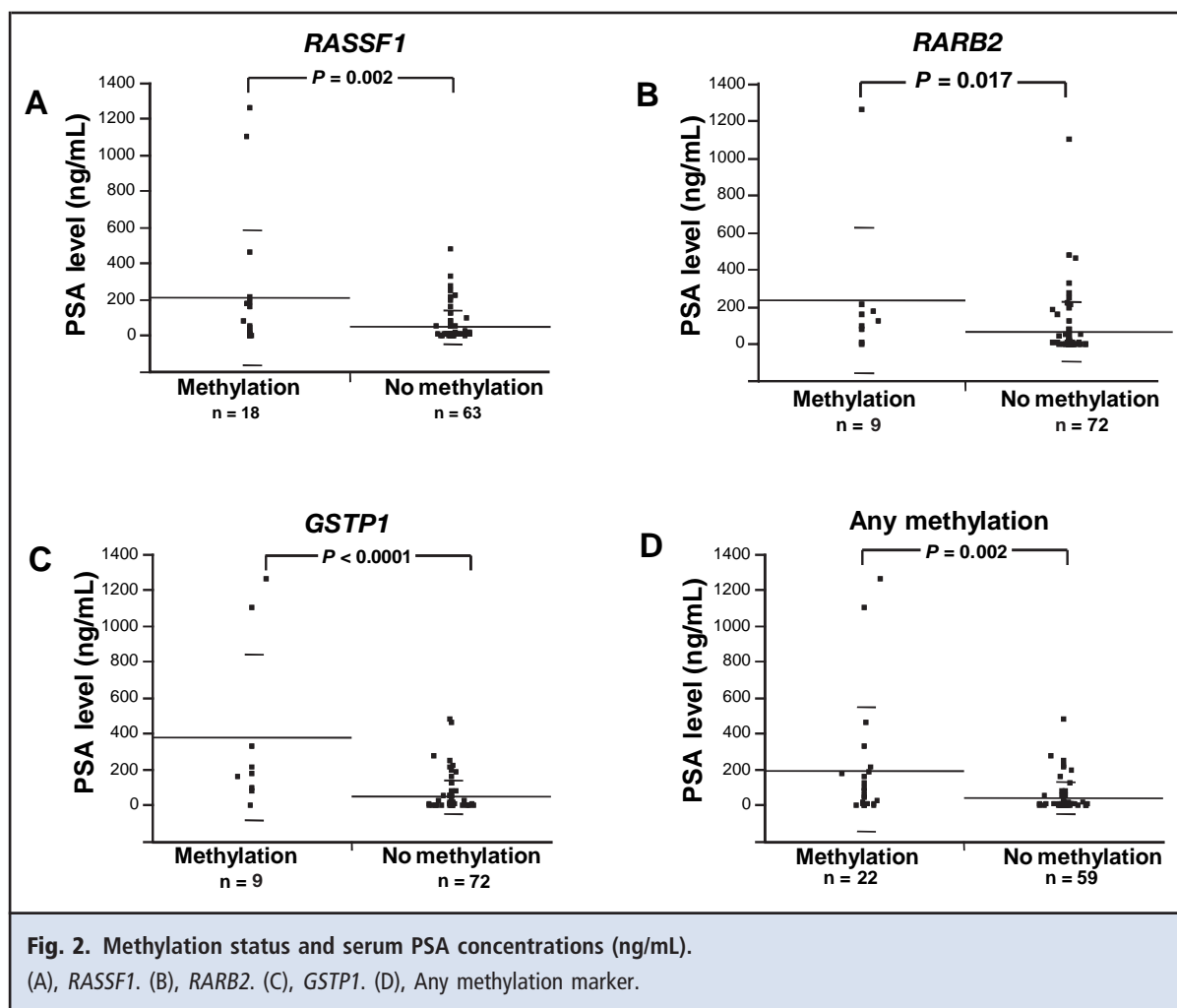**CORRELATION OF GLEASON SCORE WITH CIRCULATING DNA LOH AND METHYLATION**

Primary PCa tumor Gleason score also correlated with AI and methylation status. The Gleason score was available in 70 (84%) of 83 patients. There were no significant differences between the mean Gleason score in patients with AI of D9S171, D8S261, D6S286, D8S262, and D18S70 and that of heterozygous patients. The mean PCa Gleason score in patients with hypermethylated *RASSF1*, *RARB2*, and *GSTP1* was significantly higher (*P* = 0.017, 0.042, and 0.016, respectively) than in patients without hypermethylated DNA. Patients with ≥1 hypermethylated circulating tumor-related gene had tumors with significantly higher Gleason scores than patients without hypermethylated DNA (*P* = 0.019; Table 2).

CORRELATION OF AJCC STAGE WITH CIRCULATING DNA METHYLATION

Detection of circulating methylated tumor-related DNA also correlated with AJCC PCa stage. Circulating methylated DNA of *RARB2* and *GSTP1* in serum was more common with increasing AJCC stage. Stage IV patients showed significantly more frequent hypermethylation of *RARB2* and *GSTP1* than stage I, II, and III patients (*P* = 0.027 and 0.019, respectively). Circulating methylated DNA of *RARB2* and *GSTP1* was not seen in stage I, II, or III patients (Table 3).

COMBINATION OF CIRCULATING LOH AND METHYLATED DNA

Neither LOH nor hypermethylated DNA markers alone were able to identify all patients with PCa. By combining the 2 circulating DNA assays, the number of PCa patients positive for ≥1 methylation or LOH marker increased to 52 of 83 (63%). Of the 81 patients with available serum PSA (closest analysis to blood draw for DNA assays), 24 (30%) had concentrations considered to be at low risk for a PCa diagnosis (<4 μg/L). By combining the MSP and LOH assays, we identified 15 of 24 (63%) PCa cases not detected by

Fig. 2. Methylation status and serum PSA concentrations (ng/mL).
(A), *RASSF1*. (B), *RARB2*. (C), *GSTP1*. (D), Any methylation marker.

PSA concentration alone (Fig. 3A). The dual platform multimarker assays detected circulating tumor-related DNA with similar sensitivity, regardless of AJCC stage. Methylation or LOH of circulating tumor-related DNA was detected in 18 of 25 (72%) stage I/II/III and 34 of 58 (59%) stage IV PCa patients (Fig. 3B). In 57 (70%) of the 81 PCa patients, PSA >4 μg/L was detected. Overall, the combination of the DNA assays and PSA assay positivity in the analysis of 81 PCa patients gave a sensitivity of 89%.

Discussion

The evaluation of serum for PCa-associated genetic and epigenetic changes has several advantages. Unlike tissue biopsy, blood sampling is minimally invasive, has little or no morbidity, and can be repeated to monitor changes in disease or detect recurrence. Additionally, the use of multiple molecular markers based on appropriate tumor-related genes minimizes the false-positive rate [19]. Currently, the only blood-based PCa screening method is serum PSA measurement, which is limited in its utility because of the high rate of false positives and false negatives [3–6].

Our study of circulating DNA from patients with PCa examined 6 AI markers associated with chromosomes 6q, 8p, 9p, 10p, and 18q. In primary PCa tissue, AI has been reported on these chromosomes [13, 20–26] at frequencies that vary with tumor stage/Gleason score and with assay parameters; however, very few studies have examined AI in body fluid specimens from patients with PCa. A recent report demonstrated 0%–18% rates of LOH for various chromosomal loci in plasma DNA; 44% of specimens tested positive for at least 1 of 15 LOH markers [27]. By comparison, 47% of analyzed serum specimens in our study tested positive for at least 1 of 6 LOH markers. The similar rates of positive markers in these 2 independent multimarker studies suggest that LOH may be detecting patients having a specific clinical status.

Table 2. Serum methylation and AI biomarkers vs Gleason score.

DNA marker	Mean Gleason score (n)	P
RASSF1		
Methylated	8.1 (17)	0.017
Unmethylated	7.3 (53)	
RARB2		
Methylated	8.2 (9)	0.042
Unmethylated	7.3 (61)	
GSTP1		
Methylated	8.3 (9)	0.016
Unmethylated	7.3 (61)	
Any methylation marker		
Methylated	8 (20)	0.019
Unmethylated	7.2 (50)	
D6S286		
AI	7.3 (12)	NS[a]
R[b]	7.3 (26)	
D8S261		
AI	8.2 (6)	NS
R	7.5 (18)	
D8S262		
AI	6.9 (9)	NS
R	7.6 (23)	
D9S171		
AI	7.8 (8)	NS
R	7.6 (29)	
D10S591		
AI	7.3 (9)	NS
R	7.7 (28)	
D18S70		
AI	7.2 (10)	NS
R	7.6 (42)	
Any LOH		
AI	7.5 (33)	NS
R	7.4 (35)	

[a] NS, not significant.
[b] R, allele retention.

Table 3. Methylation status and AJCC stage.

Serum DNA marker and methylation status	AJCC stages I–III, n (%) (n = 25)	AJCC stage IV, n (%) (n = 58)	P
RASSF1			
Methylated	4 (16)	16 (28)	NS[a]
Not methylated	21 (84)	42 (72)	
RARB2			
Methylated	0 (0)	10 (17)	0.027
Not methylated	25 (100)	48 (83)	
GSTP1			
Methylated	0 (0)	11 (19)	0.019
Not methylated	25 (100)	47 (8)	
Any methylation marker			
Methylated	4 (16)	20 (35)	NS
Not methylated	21 (84)	38 (66)	

[a] NS, not significant.

in circulating DNA. We found that *GSTP1*, *RASSF1*, and *RARB2* were hypermethylated in 13% (11 of 83), 24% (20 of 83), and 12% (10 of 83) of serum specimens from PCa patients, respectively. In combination, the 3 markers identified 29% of patients with PCa.

Fig. 3. Combination of circulating LOH and methylated DNA biomarkers.

Comparison of all patients and patients with PSA ≤4 μg/L (A) and AJCC stages I–III and stage IV (B).
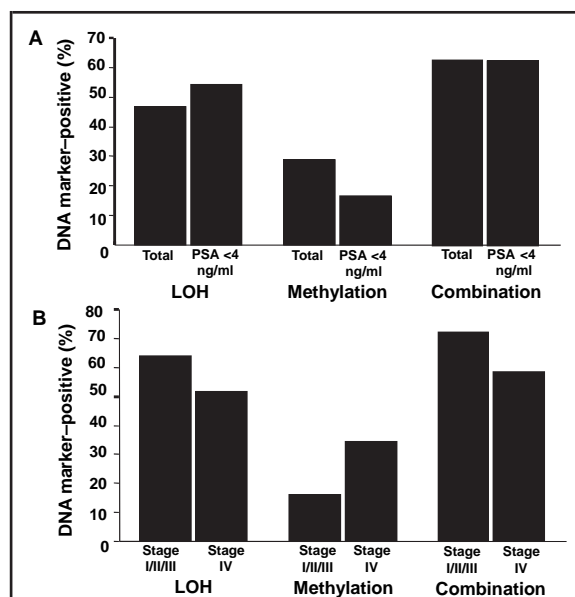
Our study also examined hypermethylation of the promoter region for 3 tumor-related genes. *GSTP1*, *RASSF1*, and *RARB2* are reportedly hypermethylated in 36%–99%, 53%–96%, and 53%–75% of PCa tissue specimens, respectively *(28–34)*. *GSTP1* is hypermethylated in 15%–52% of serum specimens from patients with PCa *(38, 39)*, but there are no published reports on hypermethylation of *RASSF1A* and *RARB2*

A DNA marker's half-life and clearance from the bloodstream may impact its clinical utility. Similar to other malignancies, PCa is characterized by genetic and epigenetic heterogeneity, particularly during tumor progression *(13, 23, 24, 29, 30)*. Hoque et al. *(40)* used a 9-marker panel to detect promoter region hypermethylation in DNA obtained from the urine sediment of PCa patients. Although 5 of these markers were also positive in the urine of normal controls, the authors proposed that a combination of *CDKN2A* [cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4); alias, *p16*], *ARF* (ADP-ribosylation factor), *MGMT* (O-6-methylguanine-DNA methyltransferase*),* and *GSTP1* could theoretically identify 87% of patients with PCa. While these results are impressive, their diagnostic and prognostic utility remains to be assessed.

The average DNA amount extracted from serum of healthy donors was about 260 μg/L *(17)* using our method. The amount of DNA extracted from the sera of prostate cancer patients was 2- to 3-fold higher than the amount from healthy donors. Therefore, for methylation analysis, lack of hypermethylation meant that not only could we not detect any methylation peak, but also that an unmethylated peak could be detected by CAE; the latter is a built-in control for the presence of DNA. In LOH analysis of circulating DNA, the detection of LOH is more difficult to assess because circulating DNA from cancer patients contains DNA from both normal (no LOH) and cancer cells (with LOH). In our study, neither hypermethylation nor LOH was detected in circulating DNA from normal donors, confirming the high specificity and positive predictive value of our dual-platform assay. To be precise, the number of normal donors assessed was 40, so at a 95% CI the assay has specificity between 91% and 100%. We attempted to bring clinical relevance to our assay by correlating genetic and epigenetic changes to known diagnostic and prognostic factors, such as Gleason score and AJCC stage, which are independent of serum. We could not detect a significant correlation between Gleason score and any AI; however, each methylation-positive group (*RASSF1, RARB2, GSTP1*, and any 1 methylated marker) showed significantly higher mean Gleason scores than each methylation-negative group ($P = 0.017$, $P = 0.042$, $P = 0.016$, and $P = 0.02$, respectively). The correlation of higher mean Gleason scores to methylation of tumor-related genes indicates that more advanced or aggressive tumors were likely to harbor these epigenetic aberrations.

We could not detect a significant correlation between PSA concentration and any AI; however, each methylation-positive group (*RASSF1, RARB2, GSTP1*, and any 1 methylated marker) showed significantly higher mean PSA concentrations ($P = 0.002$, $P =$ 0.017, $P < 0.001$, and $P = 0.002$, respectively). These patients represent the portion of PCa cases that may be missed if diagnosis is based on serum PSA in the absence of a palpable mass on digital rectal examination. Among the 30% of patients with a normal serum PSA concentration (<4 μg/L), 54% had at least 1 positive microsatellite marker, 17% had at least 1 positive methylation marker, and 63% tested positive for PCa based on dual-platform assay criteria.

AI was independent of AJCC stage of PCa, possibly because AJCC staging considers nodal and distant metastasis as well as the primary tumor. Thus the primary tumor of a patient with AJCC stage I/II PCa may have a high Gleason score. Also, tissue studies show that AI is often independent of AJCC T-stage but may be inversely linked to invasive potential *(23, 29)*. In our study, no single LOH marker identified all patients with PCa, and no combination of LOH markers identified more than 47% of cases. In contrast, the frequency of hypermethylation increased directly with AJCC stage of disease. The observation that *RARB2* and *GSTP1* were methylated only in patients with stage IV disease is consistent with a putative role for these genes in invasion and metastasis.

As hypothesized, the combination of LOH and methylation assays was more informative than either assay alone. At least 1 marker of LOH or methylation was detected in circulating DNA from 63% of patients (52 of 83) with PCa, compared with 16% for LOH analysis alone and 34% for methylation analysis alone. As expected, the range of our dual-platform, multimarker assay compensated not only for the expected heterogeneity of tumor cells *(13, 23, 24, 29, 30)* but also for varying rates of clearance for free circulating DNA. Our approach may represent a useful complement to serum PSA for the diagnosis or surveillance of PCa. In our study, PSA alone detected 71% of PCa cases, whereas the combination of PSA, LOH, and methylation detected 89% of PCa cases without increasing the rate of false-positive results.

Several factors may improve the overall sensitivity and specificity of our dual-platform molecular assay. Serial assessment of patient blood may provide additional sensitivity and may be particularly helpful for early-stage disease and detection of recurrence. Different/additional methylation and LOH markers may increase the assay's ability to accommodate tumor heterogeneity and differences in the clearance rates of various lengths of circulating tumor-related DNA. Although the assay had limited correlation with known diagnostic and prognostic factors of PCa, its sensitivity in patients with early-stage disease merits its incorporation into prospective treatment protocols, where it can be investigated as a monitoring tool. The assays may be of clinical utility in monitoring patients re-

ceiving treatment or those with potentially aggressive disease.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.
2. Greene FL, Page DL, Fleming ID, Fritz A, Balch CM, Haller DG, Morrow M. AJCC cancer staging manual. 6th ed. New York: Springer-Verlag; 2002. 421 p.
3. Beatty PA. Prostate cancer with low PSA levels. N Engl J Med 2004;351:1802–3.
4. Bock JL, Klee GG. How sensitive is a prostate-specific antigen measurement? How sensitive does it need to be? Arch Pathol Lab Med 2004; 128:341–3.
5. Pfister C, Basuyau JP. Current usefulness of free/total PSA ratio in the diagnosis of prostate cancer at an early stage. World J Urol 2005;23:236–42.
6. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. N Engl J Med 2004;350:2239–46.
7. American Cancer Society. Prostate cancer: early detection. http://www.cancer.org/docroot/CRI/content/CRI_2_6x_Prostate_Cancer_Early_Detection.asp?sitearea=&level (Accessed January 2009).
8. Dahiya R, Lee C, McCarville J, Hu W, Kaur G, Deng G. High frequency of genetic instability of microsatellites in human prostatic adenocarcinoma. Int J Cancer 1997;72:762–7.
9. Dong JT, Boyd JC, Frierson HF Jr. Loss of heterozygosity at 13q14 and 13q21 in high grade, high stage prostate cancer. Prostate 2001;49: 166–71.
10. Karan D, Schmied BM, Dave BJ, Wittel UA, Lin MF, Batra SK. Decreased androgen-responsive growth of human prostate cancer is associated with increased genetic alterations. Clin Cancer Res 2001;7:3472–80.
11. Karan D, Lin MF, Johansson SL, Batra SK. Current status of the molecular genetics of human prostatic adenocarcinomas. Int J Cancer 2003;103: 285–93.
12. Canto EI, Shariat SF, Slawin KM. Molecular diagnosis of prostate cancer. Curr Urol Rep 2004;5: 203–11.
13. von Knobloch R, Konrad L, Barth PJ, Brandt H, Wille S, Heidenreich A, et al. Genetic pathways and new progression markers for prostate cancer suggested by microsatellite allelotyping. Clin Cancer Res 2004;10:1064–73.
14. Konishi N, Nakamura M, Kishi M, Ishida E, Shimada K, Matsuyoshi S, et al. Genetic mapping of allelic loss on chromosome 6q within heterogeneous prostate carcinoma. Cancer Sci 2003;94: 764–8.
15. Harden SV, Sanderson H, Goodman SN, Partin AA, Walsh PC, Epstein JI, Sidransky D. Quantitative GSTP1 methylation and the detection of prostate adenocarcinoma in sextant biopsies. J Natl Cancer Inst 2003;95:1634–7.
16. Singal R, Ferdinand L, Reis IM, Schlesselman JJ. Methylation of multiple genes in prostate cancer and the relationship with clinicopathological features of disease. Oncol Rep 2004;12:631–7.
17. Mori T, O'Day SJ, Umetani N, Martinez SR, Kitago M, Koyanagi K, et al. Predictive utility of circulating methylated DNA in serum of melanoma patients receiving biochemotherapy. J Clin Oncol 2005;23:9351–8.
18. Fujimoto A, O'Day SJ, Taback B, Elashoff D, Hoon DS. Allelic imbalance on 12q22–23 in serum circulating DNA of melanoma patients predicts disease outcome. Cancer Res 2004;64:4085–8.
19. Mori T, Martinez SR, O'Day SJ, Morton DL, Umetani N, Kitago M, et al. Estrogen receptor-alpha methylation predicts melanoma progression. Cancer Res 2006;66:6692–8.
20. Cooney KA, Wetzel JC, Consolino CM, Wojno KJ. Identification and characterization of proximal 6q deletions in prostate cancer. Cancer Res 1996;56: 4150–3.
21. Verhagen PC, Hermans KG, Brok MO, van Weerden WM, Tilanus MG, de Weger RA, et al. Deletion of chromosomal region 6q14–16 in prostate cancer. Int J Cancer 2002;102:142–7.
22. Lu W, Takahashi H, Furusato B, Maekawa S, Ikegami M, Sudo A, et al. Allelotyping analysis at chromosome arm 8p of high-grade prostatic intraepithelial neoplasia and incidental, latent, and clinical prostate cancers. Genes Chromosomes Cancer 2006;45:509–15.
23. Matsuyama H, Pan Y, Oba K, Yoshihiro S, Matsuda K, Hagarth L, et al. Deletions on chromosome 8p22 may predict disease progression as well as pathological staging in prostate cancer. Clin Cancer Res 2001;7:3139–43.
24. Perinchery G, Bukurov N, Nakajima K, Chang J, Li LC, Dahiya R. High frequency of deletion on chromosome 9p21 may harbor several tumor-suppressor genes in human prostate cancer. Int J Cancer 1999;83:610–4.
25. Trybus TM, Burgess AC, Wojno KJ, Glover TW, Macoska JA. Distinct areas of allelic loss on chromosomal regions 10p and 10q in human prostate cancer. Cancer Res 1996;56:2263–7.
26. Jenkins R, Takahashi S, DeLacey K, Bergstralh E, Lieber M. Prognostic significance of allelic imbalance of chromosome arms 7q, 8p, 16q, and 18q in stage T3N0M0 prostate cancer. Genes Chromosomes Cancer 1998;21:131–43.
27. Schwarzenbach H, Chun FK, Lange I, Carpenter S, Gottberg M, Erbersdobler A, et al. Detection of tumor-specific DNA in blood and bone marrow plasma from patients with prostate cancer. Int J Cancer 2007;120:1465–71.
28. Liu L, Yoon JH, Dammann R, Pfeifer GP. Frequent hypermethylation of the RASSF1A gene in prostate cancer. Oncogene 2002;21:6835–40.
29. Woodson K, Gillespie J, Hanson J, Emmert-Buck M, Phillips JM, Linehan WM, Tangrea JA. Heterogeneous gene methylation patterns among preinvasive and cancerous lesions of the prostate: a histopathologic study of whole mount prostate specimens. Prostate 2004;60:25–31.
30. Maruyama R, Toyooka S, Toyooka KO, Virmani AK, Zochbauer-Muller S, Farinas AJ, et al. Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clin Cancer Res 2002;8:514–9.
31. Kang GH, Lee S, Lee HJ, Hwang KS. Aberrant CpG island hypermethylation of multiple genes in prostate cancer and prostatic intraepithelial neoplasia. J Pathol 2004;202:233–40.
32. Nakayama T, Watanabe M, Yamanaka M, Hirokawa Y, Suzuki H, Ito H, et al. The role of epigenetic modifications in retinoic acid receptor beta2 gene expression in human prostate cancers. Lab Invest 2001;81:1049–57.
33. Enokida H, Shiina H, Urakami S, Igawa M, Ogishima T, Li LC, et al. Multigene methylation analysis for detection and staging of prostate cancer. Clin Cancer Res 2005;11:6582–8.
34. Bernardini S, Miano R, Iori R, Finazzi-Agro E, Palmieri G, Ballerini S, et al. Hypermethylation of the CpG islands in the promoter region of the GSTP1 gene in prostate cancer: a useful diagnostic and prognostic marker? Clin Chim Acta 2004; 350:181–8.
35. Muller I, Beeger C, Alix-Panabieres C, Rebillard X,

Pantel K, Schwarzenbach H. Identification of loss of heterozygosity on circulating free DNA in peripheral blood of prostate cancer patients: potential and technical improvements. Clin Chem 2008; 54:688–96.

36. Hoon DS, Spugnardi M, Kuo C, Huang SK, Morton DL, Taback B. Profiling epigenetic inactivation of tumor suppressor genes in tumors and plasma from cutaneous melanoma patients. Oncogene 2004;23:4014–22.

37. Umetani N, de Maat MF, Mori T, Takeuchi H, Hoon DS. Synthesis of universal unmethylated control DNA by nested whole genome amplification with phi29 DNA polymerase. Biochem Biophys Res Commun 2005;329:219–23.

38. Jeronimo C, Usadel H, Henrique R, Silva C, Oliveira J, Lopes C, Sidransky D. Quantitative GSTP1 hypermethylation in bodily fluids of patients with prostate cancer. Urology 2002;60:1131–5.

39. Ellinger J, Haan K, Heukamp LC, Kahl P, Buttner R, Muller SC, et al. CpG Island hypermethylation in cell-free serum DNA identifies patients with localized prostate cancer. Prostate 2008;68: 42–9.

40. Hoque MO, Topaloglu O, Begum S, Henrique R, Rosenbaum E, Van Criekinge W, et al. Quantitative methylation-specific polymerase chain reaction gene patterns in urine sediment distinguish prostate cancer patients from control subjects. J Clin Oncol 2005;23:6569–75.